DOURSON, 1998

Toxicology Excellence for Risk Assessment

TERA *a nonprofit corporation dedicated to the best use of toxicity data for risk values*

MOUSE MICRONUCLEUS TEST FOR PERCHLORATE & LITERATURE REVIEW OF RELEVANT MOUSE STUDIES

Issue

Concern has been expressed that the negative results of the recently completed mouse micronucleus test (Sharma and Gao, 1998) may not be credible because the top dose of 1000 mg/kg-day might not have been sufficiently high. This study tested oral gavage doses of either 0, 62.5, 125, 250, 500, or 1000 mg/kg-day to 5 mice per sex over 3 days.

Response

The primary approach to this issue was to obtain the results of the clinical findings of the dose-range finding study which preceded the definitive mouse micronucleus test, as well as the clinical findings of the definitive test (Sharma and Gao. 1998). This would allow a determination of whether the negative results of the definitive test could be accepted with confidence. An alternative, and perhaps less useful, approach to this issue was an additional literature search for mouse studies with perchlorate in order to determine the relative ranges of doses and expected responses. ManTech has also responded to this issue by way of an attachment to a letter of M. Anderson of ManTech to M. Dourson of *TERA* dated August 25, 1998, and specifically attachment 2 to this letter regarding ManTech's response to EPA comments. The response is attached to this report.

The Primary Approach

TERA reviewed the clinical data for both the dose-range finding study, and the definitive micronucleus test (Sharma and Gao. 1998). The dose-range finding study tested 3 mice per sex at doses of either 0, 500, 1000, 2000, or 4000 mg/kg-day for each of 3 days. Mortality only occurred in this study at 2000 mg/kg-day (4 out of 6 animals died), and at 4000 mg/kg-day (5 out of 6 animals died) on the first day. Animals surviving these high doses were continued at a lower dose. Plots of these mortality data are shown in figures 1 and 2. These plots show possible trends using both linear and polynomial regressions, and using both linear-linear and log-linear scales. All of these plots suggest that approximately 10 to 20% mortality might be expected from a single perchlorate dose of 1000 mg/kg-day, the top dose chosen for the definitive study.

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In the definitive study, single doses of either 0, 62.5, 125, 250, 500, or 1000 mg/kg-day were repeatedly gavaged for three days to 5 mice per sex. Clinical signs or body weight changes were not apparent in any dose of the definitive study. Nor was any mortality observed, despite the fact that based on the regressions determined from the dose-range study, 1 or 2 mice would have been expected to die at the 1000 mg/kg-day dose. The results of the subsequent micronucleus tests were negative.

The Literature Review

TERA conducted an extended literature review for any studies where mice have been given perchlorate. Several studies were found with doses in the range of the definitive micronucleus test (i.e., either 62.5, 125, 250, 500, or 1000 mg/kg-day) or higher as shown in Table 1. NOAELs and LOAELs are specified when possible.

The most relevant studies for comparison with the micronucleus test are probably those of Gauss, 1972b, Larsson-Nyren, 1996, Thorp, 1976, Connel et al., 1983 and perhaps that of Gauss, 1972a. These studies show the results of short term tests at doses that fall close to the range tested by Sharma and Gao. (1998). Some of the results of these studies are difficult to classify as either NOAELs or LOAELs. Further work could be done with thyroid experts on this issue if needed.

Gauss (1972b) has an inconsistency between the methods and results sections of the paper. The methods clearly state that perchlorate was administered in both diet and drinking water, but the results do not mention the drinking water exposure. The fully translated text is enclosed to help in this interpretation. Gauss (1972a) also appears to have this same inconsistency, but the full translation has not yet been pursued.

Summary

Resolution of the issue, that the negative results of the recently completed mouse micronucleus test (Sharma and Gao, 1998) may not be credible because the top dose of 1000 mg/kg-day might not have been sufficiently high, depends on judgment. The likelihood that mortality would be invoked in another micronucleus test at slightly higher doses, for examples doses of 1200 and 1500 mg/kg-day, seems high based on the analysis of figures 1 and 2. Table 1 shows effects both below and above the 1000 mg/kg-day dose of the micronucleus test of Sharma and Gao (1998). Whether these effects can be judged as adverse also depends on judgment, and may in fact be aided in part on how similar effects from the ongoing studies of perchlorate are being judged.

Michael J. Dourson 10·12·98

Table 1. Mouse studies with Perchlorate from a Literature Review. Table Organized by Increasing Dose of Administered Compound.

Study	Findings	NOAEL mg/kg-d	LOAEL mg/kg-d
Middlesworth, 1985	0.032 mg of potassium perchlorate per gram of low-in-iodine feed to weanling through adult CD-1 male mice from 10 to 80 days showed statistically significant decrease in body weight and thyroid iodine content, and statistically significant increase in absolute and relative thyroid weight at 80 days	None	~3 uses adult weight and feed consumption given by author; dose to weanling would be: ~8
Gauss, 1972b	0.25 ml of a saturated potassium perchlorate solution (1.675 g/100ml) given once subcutaneously showed a drop in thyroidal 24-hour ^{131}I storage by 21%, and manifestations of incipient hyperplasia of the parenchyma	None	140 (?) dose based on parameters provided by the authors
Larsson-Nyren, 1996	IP injections of a single dose of sodium perchlorate at levels of 10 to 300 mg/kg-day to adult female Umea-ob/ob mice resulted in only a slight, transient, reducing effect on the basal serum glucose at 300 mg/kg-day	300	None
Thorp, 1976	10 mg sodium perchlorate injected once i.p. with 125-iodide into 6 tumor bearing inbred GR/Afib mice showed ~14% of control iodide uptake in mammary tumors, ~1-2% of control iodide uptake in thyroid tissue, and ~100% control iodide uptake in skeletal and normal mammary tissue	None (?)	~300 (?) assumes .035 kg mouse bw
Connel et al., 1983	IP injection of a single dose of sodium perchlorate at a level of 10 mg to adult male TO mice resulted in a dramatic inhibition of thyroid iodide trapping after 60 minutes	None (?)	~330 to 400 (?) based on weights given by authors
Sharma and Gao. 1998	The definitive micronucleus test with three gavage doses of ammonium perchlorate (over 3 days) to young adult Swiss CD-1 mice of both sexes at either 62.5, 125, 250, 500, or 1000 mg/kg-day showed no clinical signs of toxicity nor mutagenicity	1000	None

Study	Findings	NOAEL mg/kg-d	LOAEL mg/kg-d
Gauss, 1972a	A variety of tests of different durations (1, 30, 64, 81, and 160 days) was conducted at doses of potassium perchlorate administered in the diet and drinking water (?) of adult female NMRI mice. Decreased food consumption, paralysis, skeletal deformation, exophthalmos, aolpecia, thyroid histopathology and mortality were seen.	None	~1,400* seems to be the lowest dose from diet; dose would be higher if mice were actually dosed in both diet and drinking water; text only translated in part
Gauss, 1972b	Potassium perchlorate administered for nine days in the diet and drinking water (?) of adult female NMRI mice. Inhibition of iodide uptake by the thyroid and thyroid histopathology were seen. Translation from the German is confusing between methods and results sections.	None	~1,700 seems to be the lowest dose from the diet, but the dose could be >6,000 if mice were actually dosed in both diet and drinking water
Sharma and Gao. 1998	Dose range finding study for the definitive micronucleus test with three gavage dose of ammonium perchlorate (over 3 days) to young adult Swiss CD-1 mice of both sexes at either 0, 500, 1000, 2000, or 4000 mg/kg-day showed mortality at the highest two doses on day one of exposure	1000	2000
Middlesworth, 1985	1% potassium perchlorate in drinking water to weanling CD-1 male mice for 10 days showed thyroid iodine severely depleted	None	~3000 assumes 0.01 kg bw and 0.003 L/day
Pajer and Kalisnk, 1991	46 weeks exposure of 1.2% sodium perchlorate in drinking water to female BALB/c mice showed strong hypothyroidism with hypertrophic and hyperplastic thyroid epithelial cells as well as pituitary thyrotropic cells.	None	~3200 assumes 0.03 kg bw and 0.008 L/day

*_TERA_ (1997) lists this value as 2011 mg/kg-day in its 1997 report on the proposed perchlorate reference dose (RfD) based on assumptions that were further refined here from the partial translation.

References:

Connell, JMC, MM Ferguson, DSC Chang and WD Alexander. 1983. Influence of sodium perchlorate on thiourylene antithyroid drug accumulation in mice. J. Endrocr. 98:183-187.

Gauss, W. 1972a. The behavior of some physiological and histological criteria of thyroid function with onetime or more lengthy dosages of potassium perchlorate to adult mice. I. Long term tests. (Das verhalten einiger physiologischer und histologischer kriterien der schilddrusenfunktion bei einmaliger oder langerer verabreichung von kaliumperchlorat an adulte mause I. Langzeitversuche.) Z. Mikrosk. Anat. Forsch. 4: 469-500.

Gauss, W. 1972b. The behavior of some physiological and histological criteria of thyroid function with onetime or more lengthy dosages of potassium perchlorate to adult mice. II. Short term tests. (Das verhalten einiger physiologischer und histologischer kriterien der schilddrusenfunktion bei einmaliger oder langerer verabreichung von kaliumperchlorat an adulte mause II. Kurzzitversuche.) Z. Mikrosk. Anat. Forsch. Leipzig 86:1, S.81-118.

Larsson-Nyren, G. 1996. Perchlorate is hypoglycaemic by amplifying glucose-stimulated insulin secretion in mice. Acta Physiol Scand 158:71-76.

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Pajer, Z and M Kalisnk. 1991. The effect of sodium perchlorate and ionizing irradiation on the thyroid parenchymal and pituitary thyrotropic cells. Oncology 48: 317-320.

Sharma, S and P Gao. 1998. Genotoxicity assays for ammonium perchlorate. Final Report/Study 6100-001. ManTech Environmental Technology, Inc. Research Triangle Park, North Carolina.

Thorpe, S. 1976. Increase uptake of iodide by hormone-responsive compared to hormone-independent mammary tumors in GR mice. Int. J. Cancer 18: 345-350.

Toxicology Excellence for Risk Assessment (*TERA*). 1997. Proposed Perchlorate Reference Dose (RfD). Prepared for The Perchlorate Study Group, Peer Review Draft, February 1997.

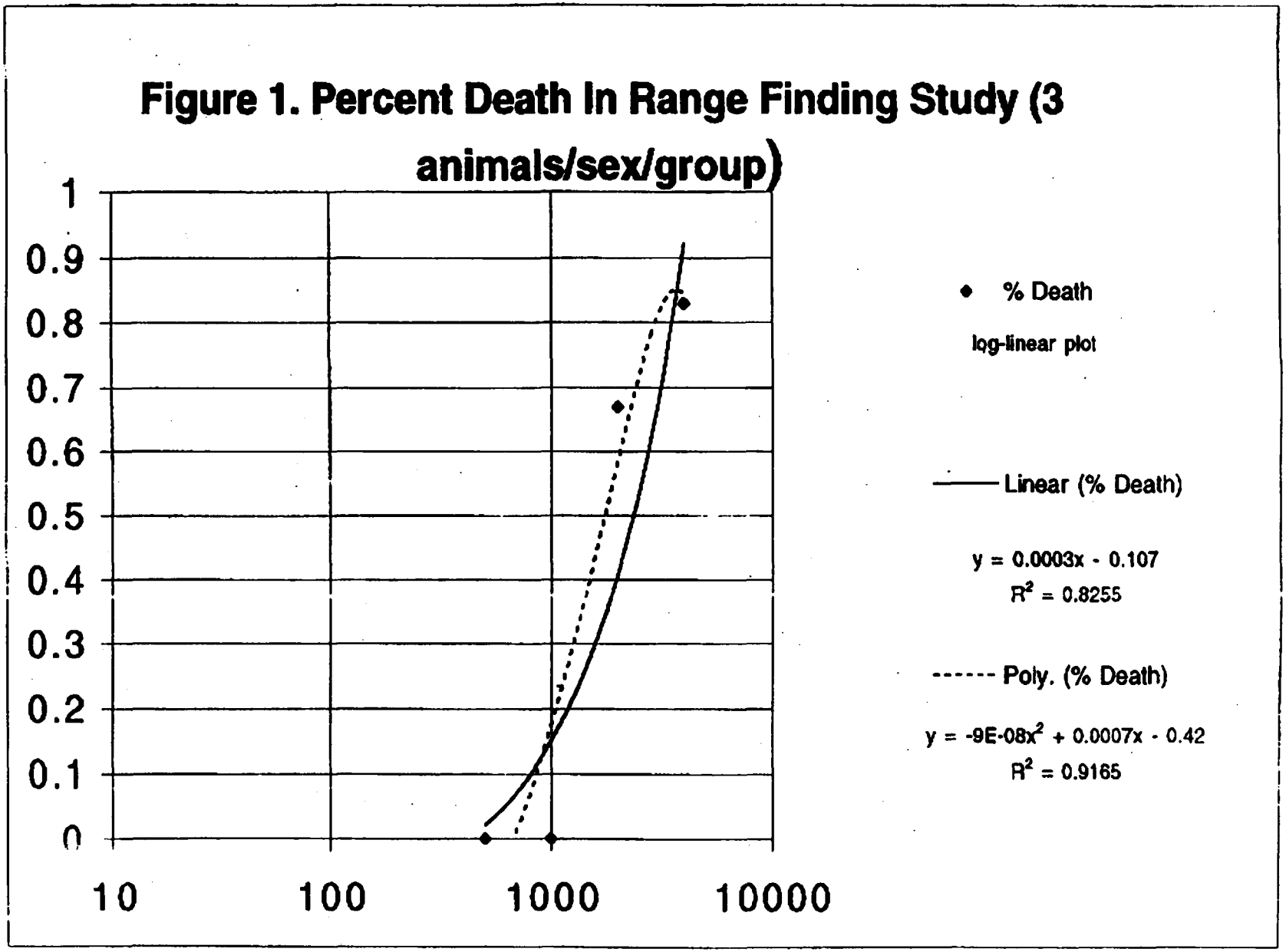
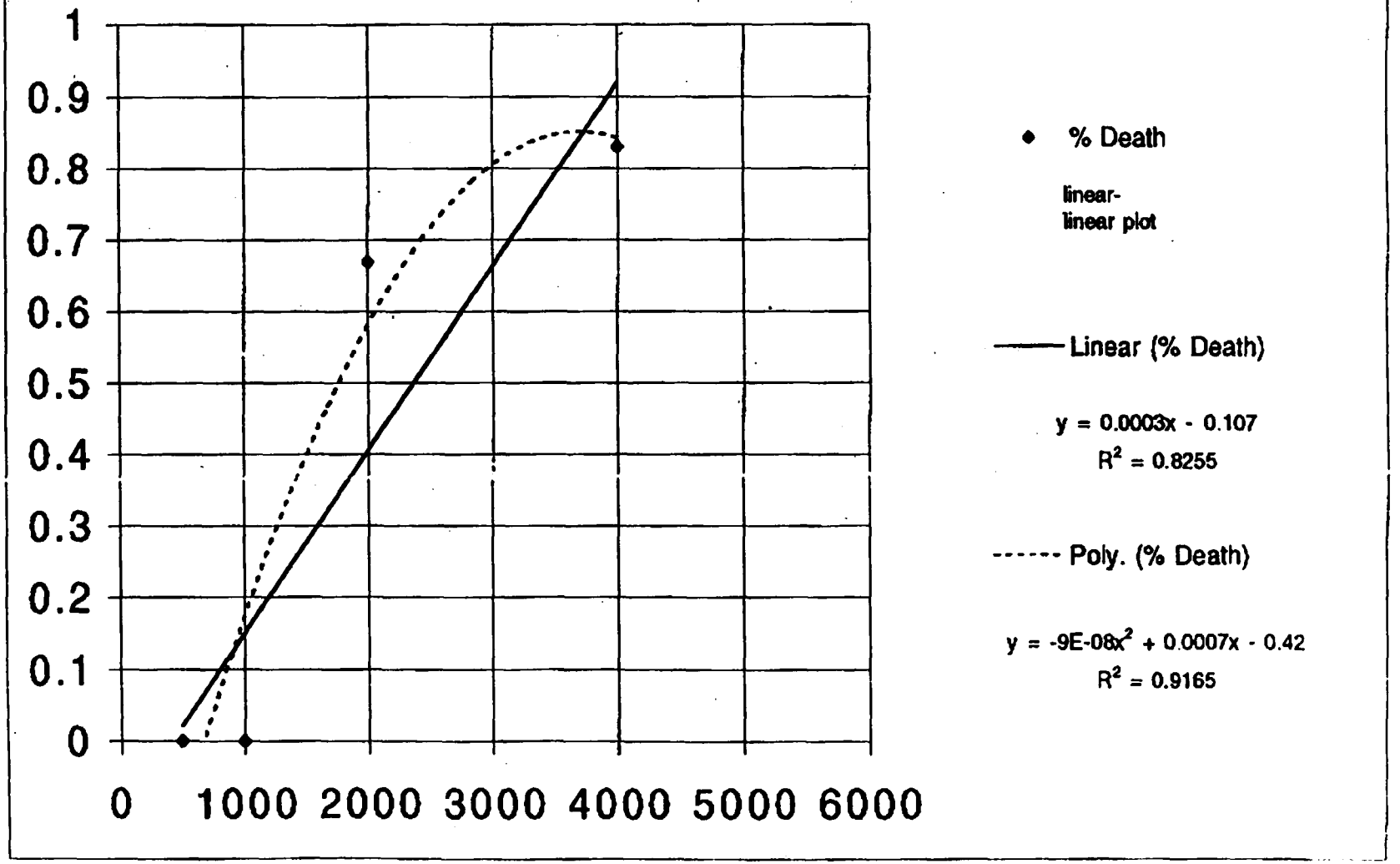


Figure 1. Percent Death In Range Finding Study (3 animals/sex/group)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
100
1000
10000
% Death
log-linear plot
Linear (% Death)
y = 0.0003x - 0.107
R2 = 0.8255
Poly. (% Death)
y = -9E-08x2 + 0.0007x - 0.42
R2 = 0.9165

Figure 2. Percent Death In Range Finding Study (3 animals/sex/group)



MANTECH ENVIRONMENTAL TECHNOLOGY, INC.

August 25, 1998

Dr. Michael Dourson
TERA
4303 Hamilton Avenue
Cincinnati, Ohio 45223

Ref: Ammonium Perchlorate Project

Dear Dr. Dourson:

The purpose of this letter is to discuss the status of the referenced research project conducted by ManTech Environmental Technology, Inc. for TERA. At your request, ManTech amended its original fixed-price agreement with TERA, to include a draft final report, at no additional cost, for the ammonium perchlorate study. During TERA's review of ManTech's draft final report, we responded to numerous questions concerning the conduct of the genotox assays performed, and provided substantial historical data and background information to TERA staff in support of our assay results, including internal quality assurance audit reports. This additional information was to a large extent incorporated into our final report to TERA. With the exception of some minor protocol changes, which improved the micronucleus assay, we have performed the requested studies in compliance with our agreement, as well as with utilizing solid scientific knowledge, processes, and procedures. However, based on our review of the EPA comments dated August 3, 1998, we conclude that TERA's customers have not received the benefit of all the discussions or the additional supporting detail that ManTech has provided to TERA.

With this letter, we are providing a response to each of the three comments provided by the EPA via a communication dated August 3, 1998, which TERA provided to ManTech on August 19, 1998. We also have attached related information submitted to TERA during review of our draft final report. We believe that our responses found in the enclosed documents adequately address each of the EPA's concerns. With respect to EPA's comments regarding potential re-testing, we have provided additional support for our dose selection in the micronucleus assay, and due to our regimen of multiple dosing, do not believe that any advantage would be gained by using yet higher doses. Regarding the mouse lymphoma testing, the positive control results with S9 activation were consistent between the validation assay and the ammonium perchlorate assay and were within historical values. Therefore, we believe no additional testing is required at this time. We have met and even exceeded our contractual agreement with TERA for our proposed research planned for this project.

ManTech Environmental Technology, Inc.
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Dr. Dourson
TERA
August 25, 1998

ManTech understands that additional research may be desired relating to this project and is willing to provide favorable pricing for any such additional work under agreement with the EPA, the USAF, or TERA. You may contact me at 919-406-2136 if you have any questions.

Sincerely,

Tara M. Anderson

Tara M. Anderson
Sr. Contract Analyst

cc: S. Sharma
J. Stikeleather

Enclosures: Att. 1: EPA Comments of August 3, 1998
Att. 2: ManTech Response to EPA Comments
Supporting Information Previously Provided:
Att. 3: ML Assay Validation, and:
Att. 4: Criteria for Accepting ML Assay

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Attachment 1

Review of the mutagenicity studies on perchlorate
Prepared by Vicki L. Dellarco
August 3, 1998

Conclusions: Ammonium Perchlorate was evaluated in Salmonella typhimurium for point mutations, in the mouse lymphoma *in vitro* assay for gene mutations, and in CD-1 mice for induction of micronuclei in bone marrow cells. This is an adequate battery of tests to determine the mutagenic and clastogenic potential of an agent. Negative results were reported in all three assays. Although perchlorate is not likely to be mutagenic, there are limitations in the reporting and in the conduct of the mouse lymphoma and mouse micronuclei assays that weakens the confidence in a negative conclusion. Thus, additional information is requested from the testing laboratory to help clarify certain issues, and it is recommended that certain assays tests be considered for repeat testing. A review of each assay follows.

A. Salmonella typhimurium (Ames Assay): TA98, TA100, TA1535, TA1538
The method and the results are acceptable. No repeat testing is recommended. Some testing labs will use both TA98 and TA100 for range finding/toxicity studies, and typically observe the appearance of the background lawn. This lab used TA100. It was uncertain from the report whether the appearance of the background lawn (which is an indicator of toxicity) was noted in the study. If this was done, was there anything remarkable?

Recommendation: No further testing needed.

B. Mouse Micronucleus Test
Based on the information contained in the report, it appears that the highest dose tested did not reach an MTD. The range finding study is only briefly described, but 2000 mg/kg appears to be about the LD50 dose. Therefore, the top dose in the micronucleus assay should have been about 75-85% of that dose, i.e, ~1500-1700 mg/kg. The highest dose was 1000 mg/kg. This is problematic given the negative responses. The testing laboratory needs to provide data that will give confidence that 1000 mg/kg is an MTD. If the 1000 mg/kg dose can not be confirmed to reach an MTD, then this study should be repeated. If this study is repeated, one should score 2000 PCE's per animal since this will increase the assay's sensitivity. Although 1000 PCE/animal is consistent with current EPA guidelines, this parameter is being changed to 2000 PCE/animal to be consistent with OECD guidelines. Another issue concerns the route of administration. If one wanted to simply answered the question of whether the agent has the ability to damage chromosomes, then the route typically considered by testing labs for this assay is i.p. or i.v., which may result in a higher dose to the bone marrow via the circulatory system.

Recommendation: Repeat bone marrow assay if testing lab can not provide data that provides confidence that an MTD was reached in study.

C. Mouse Lymphoma Assay
The results of the positive control is considered too low for the assay with S9 activation. This is problematic given that perchlorate was found to be negative in this assay. Thus, this assay needs to be repeated with S9. Also, there are some plating efficiencies that appear too high and unusual

for this assay, and thus it appears the lab had trouble with this test. When the repeat assay is done, it is strongly recommended that the testing lab should consult with Dr. Martha Moore of the NHEERL/EPA. Although there is more confidence in the negative results without S9 because of the range of doses examined, we would prefer this assay be repeated if possible.

Recommendation: Repeat test with S9 mix.

Attachment 2

Response to the EPA Review of the Mutagenicity Studies on Ammonium Perchlorate (AP)

A. Ames Assay with TA98, TA100, TA1535 and TA1537

Historically, our laboratory has always used only one strain, TA100, for range finding studies. The appearance of background lawn is always noted in a study and we have done so with this study on AP also. If a clearing of the background lawn of bacterial growth (an indication of extreme toxicity) is observed, only then it will be mentioned in the report. We have not observed any clearing of the background lawn or reduction in the number of spontaneous revertant colonies in the AP-treated cultures. We also want to point out that we have used TA1537 strain and not TA 1538 (as indicated in the EPA review) in the Ames Assay.

B. Mouse Micronucleus Test

Dose selection (range finding) for the micronucleus test is based on the following study observations:

A preliminary range finding (toxicity) testing was done with AP using 6 animals/dose group. As is shown in the summary table below, in the highest dose tested (4000 mg/kg or the maximum soluble dose), five of the six (3 males and 3 females) animals and in the 2000 mg/kg dose group, four of the six animals died after one dosing. The mouse that survived in the 4000 mg/kg dose group appeared lethargic even after 8 hours of the first dosing but recovered after 24 hours. The surviving animals (1 in the 4000 mg/kg and 2 in the 2000 mg/kg dose) in both groups were dosed for another 2 days and after 24 hours of the last dosing, they were sacrificed and the ratio of PCE and NCE were determined. There were no changes in the ratio of PCE to NCE compared to untreated control group. Since none of the doses reached an MTD level (based on the lack of toxicity observed from PCE/NCE ratio), the only option was to use the LD_{50} dose for selection of the top dose for micronucleus test.

Table 1. Preliminary Range Finding (toxicity) results from AP Study

Dose Group (mg/kg)	3 males			3 females			Ratio of* PCE/NCE
	# animals died (post dosing)			# of animals died (post dosing)			
	10 min	30 min	24 hr	10 min	30 min	24hr	
4000	1	2		1	1		0.64
2000			2			2	0.71
0 (Control)	0	0	0	0	0	0	0.65

*Determined 24 hours after the last of three dosings.

Based on the results of the range finding assay, the LD_{50} dose was calculated (even though multiple dosing was done for 3 consecutive days instead of the standard single dosing for generating an

LD_{50}) to be 1950 mg/kg which appears to be close to the value (1900 mg/kg) cited in the MSDS (received from Aldrich Chemical Co.) for AP.

Reasons for choosing 1000 mg/kg as the top dose:

(1) Multiple (3) gavage dosing was done to ensure that the highest concentration of the AP can be administered without killing the animals. Since each dose was administered consecutively for 3 days, there is a possibility that using a concentration higher (e.g. 1500 or 1700 mg/kg) than 1000 mg/kg would not necessarily give a sufficient number of surviving animals for MN analysis. For example, since a 50% mortality at 1500 mg/kg or a 56% mortality at 1700 mg/kg is possible (based on 66% mortality noticed at 2000 mg/kg) we might not have 3 surviving animals/sex to conduct the MN analysis since there are only 5 animals/sex for each dose group (EPA Testing Guidelines, 40 CFR Ch. 1, 7/97 ed.). Therefore, we chose 1000 mg/kg as the top dose to ensure that all 5 animals would survive. Further since the MN analysis will be done from 5000 PCEs/sex (instead of 2000 or 3000 PCEs at a lower survival rate) the study results (scoring a total of 10,000 PCEs) would be more reliable and statistically significant.

(2) The top dose selection is still in compliance with the EPA testing guidelines for the MN test cited in the article "MN as an index of cytogenetic damage: past, present, future" (Heddle et al, Environ. Mol. Mut., 18:277, 1991). In addition, the selection of a test agent top dose is given as 50% of LD_{50} in a review article on genetic toxicology testing strategies and data analysis, "The Principle and Methods of Toxicology" (D. Brusick, Chapter 15, page 570, 1994).

We could have also increased the number of animals per dose group to obtain a sufficient number of surviving animals, but it would have been cost prohibitive to complete this assay within the allotted budget. We would be happy to repeat the MN assay with a different top dose (after a repeat range finding assay with at least 2 doses (1500 at 1700 mg/kg) and score 2000 PCEs instead of 1000 PCE with a revised cost. Also, we want to stress that we have only followed EPA testing guidelines for scoring PCE and not OECD guidelines.

Choice of Route of Administration:

1. From our laboratory, the first choice of route for test chemical is by gavage. If the chemical characteristics do not allow a gavage dosing, i.p. injection is used (i.v. is not routinely used). Since both gavage and i.p. routes are permitted in the EPA guidelines for genotoxicity testing, we chose gavage dosing.

2. Another reason for selecting the gavage dosing is for giving multiple doses of AP to achieve the highest concentration possible without causing any mortality. By i.p. injection, even though the chemical would be absorbed directly into the blood stream and reach the target cells, the concentration is going to be less than by multiple gavage dosing. Moreover, a single i.p. injection of AP could be much more toxic even at 1000 mg/kg because of the fast absorption of an acute dose

into the blood. Consequently the total concentration of AP by a single injection for causing chromosomal damage is going to be less than the multiple dosing by gavage at a higher concentration. Multiple dosing of AP was also preferred to make sure that a potential mutagenic effect is not missed from a single dosing, as cell cycle delays can affect the response of the chemical with a different end result.

3. Another rationale for choosing gavage dosing is based on the assumption that since AP is being evaluated to determine its potential toxicity in humans, one of the possible exposure routes is probably by the oral route (Report of a serious environmental AP contamination in drinking water in the Lake Mead area in Nevada, McKinnon, 1998).

C. Mouse Lymphoma Assay

1. The results of the positive control, 3-MCA in the S9 activated system is not low according to the published literature (D. Brusick, 1994) and historical data from our laboratory. For example, in the published literature the range of mutant frequencies (MF) for 3-MCA at 4μg/ml varies from 200 -1000 x 10^{-6}. Therefore, theoretically the dose (2.5μg/ml) that we used should give a MF of 125 - 625 x 10^{-6}. Our study data at 2.5μg/ml shows a MF of 194 x 10^{-6} which is within the accepted range. Also, when we validated the ML assay (before testing AP) with positive controls (EMS and 3-MCA) 3-MCA showed a MF of 230 x10^{-6}. (See the attached data sheet for the experiment dated 1/26/98 which was provided to TERA during the reviewing of the draft final report).

2. Plating efficiencies were high in the -S9 assay. Again the average cloning efficiency of negative controls (medium/untreated and solvent/DMSO) is cited as 70-130% (Brusick, 1994). Our data for the non-activated system (-S9), which was done twice, are 72.7 ± 7.5% (medium) and 72.3 ± 7.1% (DMSO) in one experiment and 89.2 ± 8.0% (medium) and 70 ± 0.47% (DMSO) in the second experiment, showing that both the assays produced consistent results. Similarly, our ML validation experiment before testing AP. indicates that the background cloning efficiency was 82.7% for the -S9 and 106.7% for the +S9 controls. Historically, these values are acceptable.

   We have already provided some of this information to TERA earlier (see the attached criteria for accepting ML Assay) and also included in the justification for protocol changes (Appendix D-13) in the final report on AP.

Attachment 3

MLA_0126.xls

ML assay Validation

Mouse Lymphoma assay (01/26/98)
Confirmation of positive controls

Sample	VC(cts/dish) mean	std	cloning efficiency	TFT(cts/dish) mean	std	MF (x 10e-6)	RMF
Without S9							
no treatment	165.3	11.0	82.7	74.0	4.6	89.5	1
EMS (250 nl/ml)	135.0	31.6	67.5	405.0	57.7	600.0	6.7
with S9							
no treatment	213.3	14.6	106.7	76.0	1.7	71.3	1
3-MCA (2.5 ug/ml)	163.0	22.6	81.5	187.3	8.7	229.9	3.2

Calculations:
cloning efficiency: CE=(cts on VC dis/200 cells)x100%
mutation frequency: MF= cts on TFT dish/CE
relative mutation frequency: RMF=MF[treated]/MF[untreated]

Accepted range:

CE:	70 - 130%
MF (-)	20-120x10e-6

Attachment 4

Criteria for Accepting ML Assay

1. The average cloning efficiency of negative controls (medium/untreated and solvent/DMSO) is cited as 70-130% (Brusick, 1994). Our data for the non-activated system are 72.7 ± 7.5% to 89.2 ± 8.0% (medium) and 70.0 ± 0.47% to 72.3 ± 7.1% (DMSO); (see Appendix B-2 and B-2A) and for the activated system are 94.2 ± 13.2% (medium) and 89.27% (DMSO); (see Appendix B-3). Historically, these values are acceptable. Also, when we validated the ML assay with positive controls (EMS and 3-MCA), the background cloning efficiency was 82.7% for the -S9 and 106.7% for the +S9 controls. (See the attached data sheet for the experiment dated 1/26/98.)

2. An approximate fivefold increase in cell number for each of the 2 days following treatment of the experimental cultures is expected. We obtained an average of 3.5- to 5.8-fold (medium - day 1 and 2) or 3.7- to 5.0-fold (DMSO - day 1 and 2) increase in cell number in the negative controls. (See Appendix B-2, B-2A and B-3.) Historically, we have observed an average growth increase of 3.5- to 4.8-fold increase in cell number.

3. Mutation frequency: The normal range of background frequencies for assays performed with different cell stocks is cited as 20 to 120 x 10^{-6} (Brusick, 1994). Our background frequency during ML assay validation ranged from 71.3 (+S9) to 89.5 (-S9). Historically, the frequency values in untreated cultures ranged from 66 to 90 ± 8 (-S9) and 70 to 92 ± 9.4 (+S9). Even though our values in the current studies are 99.1 ± 8.9 and 96.5 ± 9.96 for nonactivated and 102.6 ± 14.4 for activated, they are still in the accepted range as cited by Brusick (1994). It is also noteworthy to mention that the background frequencies for solvent control (DMSO) in the two experiments (-S9) are much lower (51.2 - 82.04) than those for the AP-treated cultures (see Tables III-5 and III-5A).

4. A positive control is included for each of the systems (-S9 and +S9) to ensure that assay conditions are right and the induced mutation frequency is in the expected range (historically from the laboratory or from literature). The normal range of increase in mutation frequency for methylmethane sulfonate (-S9) at 10.4 µl/ml is from 2- to 8-fold (Brusick, 1994), whereas our results with ethylmethane sulfonate (a similar chemical mutagen) is from 4- to 7-fold at a much lower dose (0.25 µl/ml, or ~1/40th of the MMS dose). Our ML assay validation data and historical data show a 6.7-fold and a 4- 5.7-fold increase in mutation frequencies, respectively, compared to untreated control. 3-Methyl cholanthrene, the positive control for the activated system (+S9), shows a 2- to 10-fold increase in mutation frequency at 4.0 µg/ml (Brusick, 1994), whereas our results at a lower concentration (2.5 µg/ml) still show a twofold increase as compared to the DMSO control. Again, our ML validation data indicate a 3.2-fold increase at the same dose, and the historical data indicate a 2- to 3.4-fold increase.